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Dinucleoside tetraphosphatase from human blood cells

Purification and characterization as a high specific activity enzyme recognized by an anti-rat tetraphosphatase antibody

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Dinucleoside tetraphosphatase (Np4Nase; EC 3.6.1.17) has been purified 170 000-fold from a 30–60% ammonium sulfate fraction of a human blood cell extract. Purification included a dye–ligand affinity elution step using the inhibitor adenosine 5′-tetraphosphate. Human blood Np4Nase resembled rat liver Np4Nase, including recognition by anti-rat Np4Nase, but differed from homogeneous human leukemia Np4Nase in the 1000-fold lower specific activity of the latter. The results are discussed in relation to the potential role of diadenosine tetraphosphate (Ap4A) in the control of cell division and the turnover of Ap4A in blood.

Diadenosine tetraphosphate; Ap_4A; Dinucleoside tetraphosphatase; Human blood

1. INTRODUCTION

Ap4A is a product, substrate or regulator of purine nucleotide metabolism, DNA replication and cell cycle, heat-shock response, and platelet aggregation [1–4]. It is formed in reactions catalyzed by aminoacyl-tRNA synthetases [5,6], Ap4A-phosphorylase [7] and firefly luciferase [8]. Ap4A turnover may be catalyzed by the intracellular Np4Nase [9] or the unspecific ectoenzyme phosphodiesterase I/nucleotide pyrophosphatase (EC 3.1.4.1/EC 3.6.1.9) [10], present also in plasma [11].

Mammalian Np4Nase hydrolyzes specifically Np4Ns to the cognate nucleoside 5′-mono- and -triphosphates [9]. It has been purified to homogeneity from human leukemia cells [12] and rat liver [13] with 0.02 and 145 U/mg specific activities, respectively, which may reflect their molecular activities. Without data from normal human tissues, it is difficult to interpret the low efficiency of the leukemia enzyme. Here, we show that human blood cells contain Np4Nase which can be purified to a high specific activity, like the rat enzyme. Also, we have obtained an antibody recognizing human and rat Np4Nases.

Abbreviations: Ap3A, diadenosine 5′,5′′′-P^1,P^3-triphosphate; Ap4A, diadenosine 5′,5′′′-P^1,P^4-tetraphosphate; Np4N, dinucleoside 5′,5′′′-P^1,P^4-tetraphosphate; Np4Nase, dinucleoside tetraphosphatase (EC 3.6.1.17)

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2. MATERIALS AND METHODS

Human blood (100 ml) collected over CPD-adenine anticoagulant (63 ml) (Terumo Corp.) was used. Blood cells were pelleted in a Sorvall SS34 rotor (4 min at 4000 rpm and 4°C); resuspended in 130 ml 50 mM Tris, pH 7.5; freeze-thawed at −20°C; homogenized and centrifuged again, now at 15 000 rpm for 2 h. The supernatant was fractionated with ammonium sulfate; the 30–60% saturation fraction, dissolved in 50 mM Tris, pH 7.5, 0.5 mM EDTA (final volume 73 ml), was frozen at −20°C in 6-ml aliquots until used for the purification of Np4Nase.

The chromatographic procedures for the purification of blood Np4Nase were as described by Costas et al. [13] except that: Sephadex G-100 filtration and DEAE-cellulose chromatography were carried out in 1.2 × 90-cm and 2.2 × 6-cm columns, respectively; in the affinity elution chromatography, glycerol was not used.

Anti-rat Np_4Nase was obtained from a rabbit immunized with four 20-μg subcutaneous doses of rat liver Np4Nase. The enzyme was cut out from an SDS-PAGE gel after visualization with KCl [13] and homogenized in complete (1st dose; day 0) or incomplete Freund's adjuvant (2nd–4th doses; days 24, 53 and 83). The rabbit was bled on days 0 (control serum) and on day 97. Anti-Np4Nase titer was assayed by ELISA as in [14] with minor modifications: 1 ng rat liver Np4Nase was spotted onto nitrocellulose filters; the blocking agent was 1% gelatin; Tris substituted for phosphate in buffers; the second antibody was peroxidase-labeled goat anti-rabbit IgG (Sigma). Anti-Np4Nase titer was higher than 1:10000.

Except when indicated, other materials and methods were as described in [13].

3. RESULTS

The key to the purification of human blood cell Np4Nase to a high specific activity (Table I) was its elution from a dye–ligand affinity column with the inhibitor adenosine 5′-tetraphosphate. The final specific

Table I

Purification of human blood Np4Nase

Step	Volume (ml)	Protein (mg)	Activity (mU)	Specific activity (mU/mg)	Yield (%)
1. 30–60% ammonium sulfate fractionation	12	2840	320	0.11	100
2. Sephadex-G-100 gel filtration	76	580	220	0.38	68
3. DEAE-cellulose ion exchange	60	1.8	150	83	47
4. Cibacron blue F3G-A Sepharose 4B affinity elution	6.5	0.004	78	18500	24

The purification started from two 6-ml portions of a 30–60% ammonium sulfate fraction of a freeze-and-thaw extract of blood cells (see section 2). Those two portions were separately processed through steps 1–3 (the results reported in the table are a combination of data from both purification runs); the resulting material was pooled and submitted to step 4. Protein and activity were assayed as in [13].

activity was 18.5 U/mg, 170 000-fold higher than the starting fraction (Table I). Silver-stained SDS-PAGE and immunoblot with anti-rat Np4Nase showed one 18-kDa band like that shown by rat liver Np4Nase (Fig. 1). The latter has Np4Nase activity after renaturation [13].

The K_m value of human blood Np4Nase for Ap4A was 0.7 μM (Fig. 2), determined by analysis of complete reaction progress curves [15]. Np4Nase required Mg^{2+} as it had no activity in its absence. Maximal activity was found with 3–10 mM $MgCl_2$; 75%, 50% and 20% of maximal activity was observed with 1 mM, 0.5 mM and 0.2 mM $MgCl_2$, respectively (not shown).

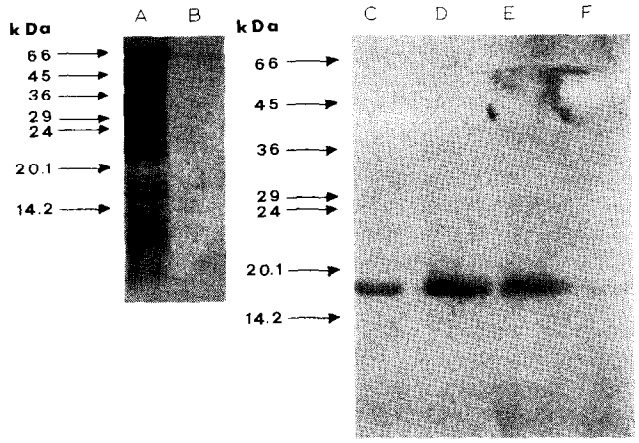

Fig. 1. Gel electrophoresis of Np4Nase. Left panel: silver-stained SDS-PAGE (15% acrylamide) analysis of human blood Np4Nase. The bands near the 66 kDa marker may correspond to known artifacts [47]. (A) 14 μg enzyme from the DEAE-cellulose step; (B) 0.15 μg from the affinity elution step (Table I). Right panel: immunoblot of (C,D) rat liver and (E,F) human blood Np4Nase. After SDS-PAGE (12.5% acrylamide) proteins were electro-transferred to a nitrocellulose membrane which was then processed by ELISA as described in section 2. Pre-immune serum gave no signal in a control immunoblot. (C) 11 μg from the DEAE-cellulose step in [13]; (D) 0.7 μg of homogeneous rat Np4Nase [13]; (E) 120 μg from the DEAE-cellulose step (Table I); (F) 0.15 μg from the affinity elution step (Table I).

Several known Np4Nase inhibitors were tested with the blood enzyme. (i) Adenosine 5′-tetraphosphate inhibits rat liver Np4Nase competitively with a 48 nM K_i value [9]. It also inhibited potently blood Np4Nase since, at 2 μM concentration, with 45 μM Ap4A as the substrate, it elicited a 42 ± 3% inhibition. This, and the 0.7 μM K_m value, correspond to a 42 nM competitive K_i value. (ii) Zn^{2+}, at micromolar concentrations, inhibits rat liver Np4Nase [16]. Fig. 3 shows a 50% inhibition of blood Np4Nase by 10 μM Zn^{2+}. Under similar conditions, rat liver Np4Nase is 50% inhibited by 2 μM Zn^{2+} [16]. (iii) Fluoride inhibits Np4Nase (0.5 mM Ap4A as the substrate) from yellow lupin seeds (50% inhibition by 2–3 μM NaF) and beef liver (50% inhibition by 20 μM NaF) [17]. We have studied the effect of NaF on human blood and rat liver Np4Nases (45 μM Ap4A as

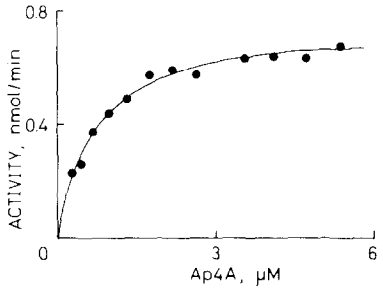

Fig. 2. Saturation kinetics of human blood NpNase. K_m = 0.7 μM; V = 0.7 nmol/min. Saturation curve and parameters were derived by analysis and computer fitting of reaction progress curves [15] recorded, at 37°C, following the hyperchromicity at 259 nm caused by the hydrolysis of Ap4A [5,8]. Completion of Ap4A hydrolysis was ensured by its irreversibility and by the coupling to alkaline phosphatase, which prevents the accumulation of reaction products ATP and AMP. The reaction was initiated by the addition of 10 μM Ap4A to a mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 μg alkaline phosphatase, and 40 ng purified blood Np4Nase. At the end of the first progress curve, a second one was recorded by repeating the addition of Ap4A; the results (not shown) were superimposable to those derived from the first progress curve (see the figure). This indicates that neither enzyme inactivation nor product inhibition interfered in the determination of saturation parameters [15].

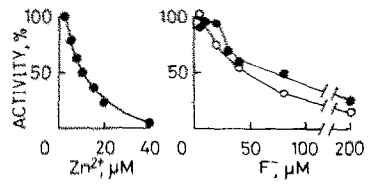

Fig. 3. Inhibition of Np4Nase by Zn^{2+} and fluoride. (•) Human blood (Table I) or (○) rat liver Np4Nase [13] from the DEAE-cellulose step were used after two desalting steps in a Sephadex G-25 column (1 × 5 cm) equilibrated in 50 mM Tris-HCl, pH 7.5. Initial rates were measured at 37°C by recording the hyperchromicity at 259 nm caused by the hydrolysis of Ap4A [5,8]. Reaction mixtures contained: 50 mM Tris-HCl, pH 7.5, 45 μM Ap4A, 1 mM $MgCl_2$, and either zinc acetate or sodium fluoride as indicated.

the substrate): 50% inhibition was caused by 80 μM and 45 μM NaF, respectively (Fig. 3).

4. DISCUSSION

The enzyme purified from human blood cells is very similar to rat liver Np4Nase [9,13], at least referring to: M_r by SDS-PAGE; K_m value for Ap4A; Mg^{2+} effect; inhibition by adenosine 5′-tetraphosphate, Zn^{2+} and fluoride; behavior during purification (including affinity elution by adenosine 5′-tetraphosphate); recognition by anti-rat Np4Nase; and specific activity of the final, (near-)homogeneous preparation.

The high specific activity of purified, human blood Np4Nase (Table I) confirms that the reportedly homogeneous, human leukemia Np4Nase is relatively very inefficient because its specific activity is 1000-fold lower [12]. This could favor high Ap4A levels in leukemia cells: a possibility related to the hypothesis that Ap4A might have a role in DNA synthesis and/or cell proliferation [1,4]. Ap4A is a primer for DNA polymerase-α [18–20] and a ligand of a polymerase-associated protein [18,21]. Evidence concerning the relationship between Ap4A levels and the rate of cell proliferation or the initiation of S-phase DNA replication is contradictory: some results favor a direct relationship [22–27]; others do not [28–32]. The effect of Ap4A might be cell type-dependent. In this regard, Np4Nase from mouse ascites tumor cells may be different to leukemia Np4Nase, because the former, after a partial purification equivalent to the first three steps of Table I, shows a specific activity of 0.3 U/mg [33], i.e. 3-fold higher than blood Np4Nase at the same level of purification (Table I) and 20-fold higher than homogeneous leukemia Np4Nase [12].

Ap4A and its homologue Ap3A have effects on blood and vascular functions. They are stored in blood platelets and released upon thrombin-induced aggregation [34–36]. Ap3A is pro-aggregatory, whereas Ap4A inhibits aggregation [35] and is a potential anti-thrombotic agent [37]. Ap4A and Ap3A have vasomotor activity and effects on hepatic cell functions [38,39]. The turnover of plasma Ap4A and Ap3A may be carried out by circulating [11,40–42] and ectoenzymatic [10,43,44] hydrolases. Circulating Ap4AC(Ap3A)-hydrolases are related to the unspecific phosphodiesterase I/nucleotide pyrophosphatase, an enzyme studied with dinucleoside polyphosphate substrates in rat liver [10], and well-known itself as an ectoenzyme in hepatocytes and other cells [45,46]. Ap4A(Ap3A)-hydrolase ectoenzyme activity in endothelial cells [43,44] probably also belongs to that class of unspecific enzymes. Np4Nase must not be confused with those unspecific Ap4A-hydrolases that may act on plasma Ap4A but are not specific for dinucleoside tetraphosphates. The specific Np4Nase is seemingly an intracellular enzyme, which makes unclear, although does not rule out, its contribution to the turnover of plasma Ap4A.

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